Transforming growth factor beta in human milk and allergic outcomes in children: a systematic review.

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Abstract

Background: Human milk (HM) transforming growth factor beta (TGF-β) is critical for inflammation regulation and oral tolerance promotion. Previous reports suggested that variations in HM TGF-β levels are associated with allergic outcomes.

Objective: We undertook a systematic review (PROSPERO 2017 CRD42017069920) to reassess the evidence on the relationships between HM TGF-β and allergic outcomes in children.

Methods: Electronic bibliographic databases (MEDLINE, EMBASE, Cochrane Library) were systematically searched. Two independent reviewers screened reference lists, extracted the data and assessed risk of bias using the National Institute for Clinical Excellence methodological checklist.

Results: A total of 21 studies were identified. Sixteen studies assessed relationships between HM TGF-β and risk of eczema; 14, allergic sensitisation; 9, wheezing/asthma; 6, food allergy; 3, allergic rhinitis/conjunctivitis. Five cohorts (5/18, 28%) reported a protective effect of TGF-β1, while 3 (3/10, 30%) suggested increased risk of allergic outcomes development and 1 (1/10, 10%), a protective effect of TGF-β2 on eczema. Meta-analysis was not possible due to significant heterogeneity in methodology, age of outcome assessment and differing statistical approaches. 71% (15/21) of studies carried a high risk of bias.

Conclusion: In contrast with previous findings we did not find strong evidence of associations between HM TGF-β and allergic outcomes. Differences in studies' methodology and outcomes do not allow unconditional rejection or acceptance of the hypothesis that HM TGF-β influences the risk of allergy development. Future studies on diverse populations employing standardised methods, accurate phenotyping of outcomes and evaluation of the effect of TGF-β in combination with other HM immune markers, microbiome and oligosaccharides are required.

Key Messages

- Basic science evidence suggests that human milk TGF-β is particularly important for oral tolerance development, with previous reports finding associations between TGF-β and immunological outcomes.
- The evidence does not support previous conclusions with most of the studies finding null associations between human milk TGF-β and allergic outcomes in children. Studies lack methodological standardisation, resulting in high heterogeneity.

- Future research should focus on the assessment of multiple immune markers, human milk oligosaccharides and microbiome in relationship with the allergic outcomes, as it is highly likely that a combination, rather than a single factor contributes to potential protective effect. Standardisation of methodology, statistical analysis and outcome definitions should be considered a top priority for the future research to allow for data meta-analysis.

Key words
Human milk; breast milk; colostrum; TGF-β; transforming growth factor-beta; allergic outcomes; allergic diseases; allergic sensitization; atopy; breastfeeding.

Abbreviations used

AR: allergic rhinitis

ARC: allergic rhinoconjunctivitis

AS: atopic sensitization

CI: confidence intervals

CMA: cow's milk allergy

ELISA: enzyme-linked immunosorbent assay

E: eczema

FA: food allergy

ISAAC: international study of asthma and allergies in childhood questionnaire

MM: mature milk

NICE: national institute for clinical excellence methodological checklists

PRISMA: preferred reporting items for systematic reviews and meta-analyses guidelines

HM: human milk

HMO: human milk oligosaccharides

OR: odds ratio

sIgE: specific immunoglobulin E

SPT: skin prick test

TGF-β: transforming growth factor-beta

TM: transitional milk

RR: Relative risk

Introduction

Human milk (HM) is the main source of nutrition during early life, a critical period of metabolic and immune programming. It is well known that HM consists of essential macro- and micronutrients, vitamins, antibodies and many other bioactive factors, essential for the growth and development of a newborn infant [1] and for protection against infections [2]. However, there is conflicting evidence on the protective role of breastfeeding in relation to the development of allergic sensitization and allergic diseases [3] with children bearing the greatest burden of these increasingly prevalent conditions in modern relatively affluent environments [4].

Transforming growth factor-beta (TGF-β) is a regulatory cytokine possessing pleiotropic functions, and is involved in physiological and pathological processes including embryogenesis, immune regulation and inflammation [5]. Three TGF-β isoforms (TGF-β1, 2 and 3) are present in HM with TGF-β2 being a predominant type [6]. TGF-β concentration varies considerably throughout lactation, with highest levels detected in colostrum [7, 8] followed by a rapid decline by 4-6 weeks of life [9-11] and a continuing decline by 3 [6, 12, 13] and 6 [14] months postpartum. There has been an increasing interest in the role of HM TGF-β as a key immunoregulatory factor that promotes IgA production [6, 15], assists with mucosal repair in the neonatal gastrointestinal tract [16], acts as a co-factor helping in the generation of immune regulatory immune responses [17] and influences the neonatal gut microbiome [18]. To date, the most comprehensive review of human studies was conducted a decade ago by Oddy and Rosales [19], reporting an association between

TGF-β levels in HM and reduced risk of immunological outcomes in children in two-thirds of the studies. The authors suggested that presence of HM TGF-β may play an important role in gut immunity functioning and maturation, leading to the subsequent promotion of oral tolerance, thus reducing the risk of allergy development [19]. High heterogeneity between the studies was highlighted, with maternal atopic status or dietary intervention during pregnancy and/or lactation suggested as the main reason.

Despite suggested immunological benefits, the role of TGF-β in allergy prevention remains controversial. Discrepant findings may be partially related to the differences in milk collection methodology, sample storage and differences in laboratory approaches. In addition, studies employed different criteria for allergic predisposition of infants, definition of outcome, method of outcome assessment, environmental influence [20] and ethnicity [21].

The importance of the links between HM composition and allergic disease development has received significant attention recently and objective assessment of existing evidence is timely. The aim of this systematic review is to summarise current knowledge on associations between HM TGF-β and atopy/allergy development.

Methods

This systematic review is reported in accordance with the recommendations set forth by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [22]. Methods were published apriori (PROSPERO 2017 CRD42017069920, available from: http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42017069920) on the 6th of July 2017).

Search strategy

An extensive electronic search of MEDLINE, EMBASE and Cochrane Library was performed on the 7th June 2017, using both free text and MESH terms. The search strategies are provided as supplementary material (**Table S1**). At a screening stage, further studies were traced through cross-checking of reference lists from identified relevant papers.

The relationships between TGF-β concentrations in HM (including colostrum, transitional milk, mature milk) and allergic diseases were studied. The primary outcome variables included atopic dermatitis/eczema, food allergy, asthma, allergic rhinitis, allergic conjunctivitis, allergic sensitization (skin prick test (SPT) and/or specific immunoglobulin E (sIgE) measurement) and serum immunoglobulin concentrations in infants and children.

Eligibility criteria and selection of articles

Studies of all designs were included if the following criteria were met: 1) Reported original data; 2) Clinical study of mother–infant dyads; 3) The study had an epidemiological design: observational studies, i.e. pregnancy cohort study, birth cohort study, human prospective study or randomized controlled trial, during pregnancy or lactation and interventional studies; 4) Included a quantitative assessment of TGF-β in HM; and 5) Investigated associations between HM TGF-β and at least one allergic disease or allergic

sensitization in the child. We excluded reviews, conference abstracts, editorials, letters to the editor, case reports and/or case series.

All included papers were transferred into EndNote reference manager. To reduce potential selection bias two independent investigators (EK and ZG) reviewed all titles and abstracts identified by the search for inclusion. Then EK and ZG independently reviewed full texts of all publications selected for data extraction. Any disagreements were resolved through discussion involving an additional reviewer (DM) and other co-authors if needed, until consensus was reached.

Data extraction

The data from each study were extracted in duplicate, tabulated, and included author and year of publication, descriptive information concerning the study design, country and setting, baseline characteristics of the study population, methodology of milk sampling, timing of sample collection, method of assessment of TGF-β concentration, outcome definition, age of outcome assessment, details on statistical analysis and overall TGF-β association with health outcome.

Quality assessment

The risk of bias was assessed in duplicate (EK and ZG) using the National Institute for Clinical Excellence (NICE) methodological checklist for cohort studies [23] and a final score was obtained by consensus.

Results

Synthesis

Based on the search strategy, a total of 353 titles were identified and 215 relevant abstracts were screened for eligibility (**FIG.1**). Of these, 26 met the inclusion criteria and were eligible for full-text assessment with 21 papers (reporting results from 20 study populations) included in our systematic review. One cohort study generated 2 publications [24, 25] and the results were summarised according to the study population, rather than by publication. Five studies were excluded as they did not assess any of the identified outcomes: 1 examined the effect of probiotic administration on the risk of eczema development and allergic sensitization only, without reporting the results on association with HM TGF-β [26], 2 studies investigated TGF-β in isolation from any allergic outcomes [27, 28], 2 studies assessed association between TGF-β and immunological but not allergic outcomes in infants [14, 15]. Due to a limited number of studies reporting immunological outcomes, the scope of this systematic review is limited to allergic outcomes. However, as our initial search included search terms relevant for immunological outcomes, these are presented in a separate subsection.

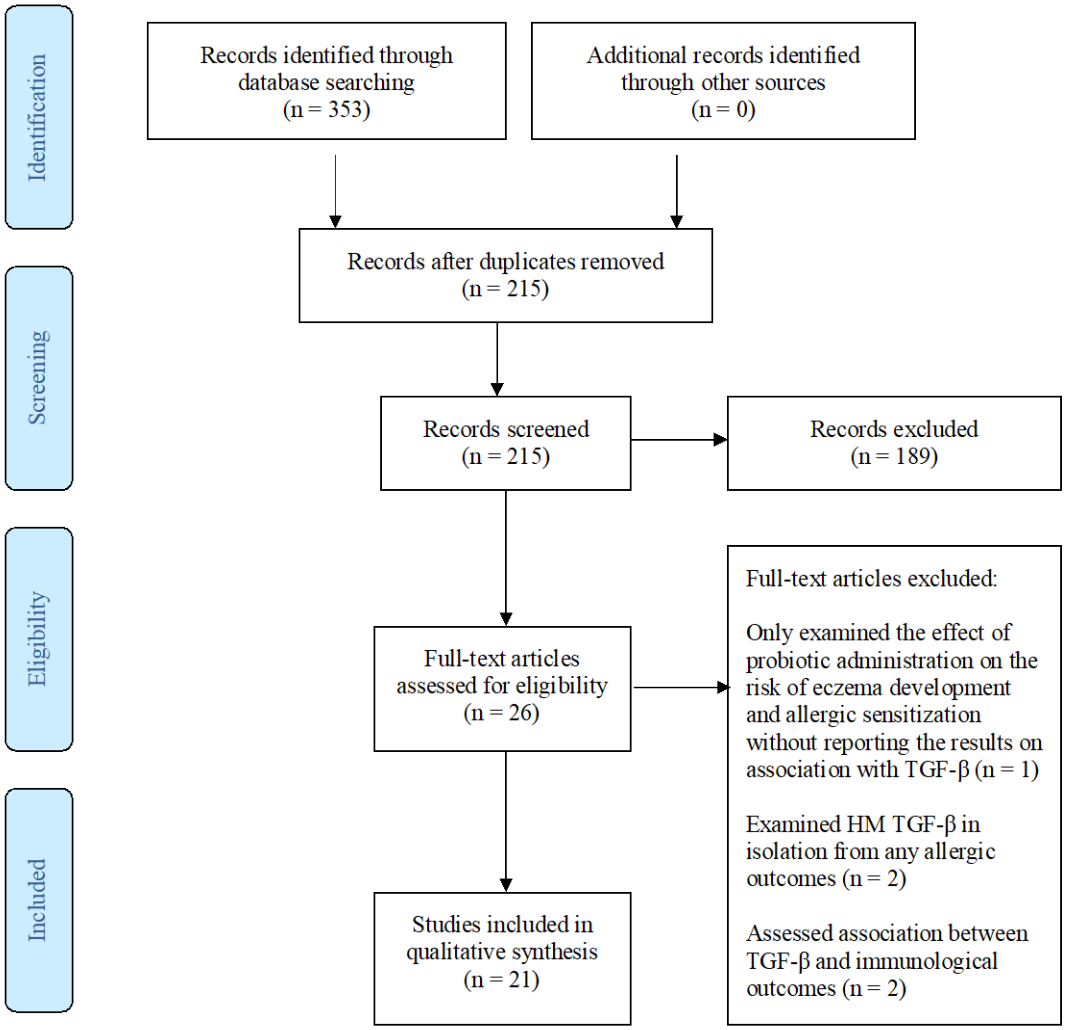


FIG.1 PRISMA diagram of the systematic search and included studies.

Description of the studies

Twenty-one studies included in this systematic review have been published between 1999 and 2017 and can be grouped into 2 main categories in accordance with the design: (a) interventional [10-13, 29-31] and (b) observational [6, 21, 24, 25, 32-41] (Table I). All included interventional studies administered either single [10, 11, 31] or multiple [12, 13, 29, 30] strains of probiotics for various durations during pregnancy [10-13] and/or lactation [29-31] with exception of 1 study, which administered either formula or pasteurized HM [39]. However, the analyses of interventional studies were undertaken within the studies without assessing the association between intervention and allergic outcomes in children. Ten studies measured TGF-β1 [11, 24, 25, 30, 33, 34, 36-

197 [39, 41], 2 investigated TGF-β2 [12, 29], 6 looked at both TGF-β1 and TGF-β2 [6, 10, 21, 31, 32, 40] and 2 studies
198 assessed all 3 TGF-β isoforms [13, 35].

199 ***Participants characteristics***

200 Study populations included participants from 15 countries, with most research conducted in Scandinavia:
201 7 studies in Finland [6, 12, 29, 31, 37, 39, 40] and 3 in Sweden [10, 21, 32]; 3 in USA [24, 25, 34, 36]; 2 in Italy [35, 38]; and 1 in
202 Australia [11], Denmark [33], Estonia [21], France [37], Germany [37], New Zealand [30], Norway [13], Russia [35],
203 Switzerland [37], The Netherlands [41] and UK [35].

204 Sample size ranged from 22 [38] to 610 participants [37] while the maximum number of HM samples reached
205 685 [12]. Most of the studies followed children up to the age of 24 months, the maximum age of follow-up
206 was 72 months [37]. Thirty-five percent of cohorts (7/20) recruited participants at high risk of allergy
207 development (Table I).

208 ***Stage of lactation***

209 Colostrum was collected within 0–4 days [6, 10, 12, 21, 29, 30, 32, 35, 38-40], transitional milk (TM) 5-14 days [11, 13,
210 36] and mature milk (MM) between 15 days and 6 months postpartum [6, 10-13, 21, 24, 25, 30-34, 38, 41]. Eight cohorts
211 [6, 10, 12, 21, 30, 32, 35, 38] measured TGF-β levels in paired colostrum and MM samples. Of the remaining cohorts,
212 3 assessed TGF-β levels in colostrum [29, 39, 40]; 1 in TM [36]; 2 in TM and MM [11, 13] and 7 in MM [24, 25, 31, 33,
213 34, 36, 37, 41].

214

215 ***Methodology of human milk samples collection and storage***

216 The time of sample collection (time of the day, pre- or post-breastfeed) differed between the studies.
217 Approaches to the sample collection varied with following sampling procedures reported: at the beginning
218 of breastfeed [12, 41], during breastfeed (from the contra-lateral breast) [35, 41], at the end of breastfeed [11]; 2
219 hours after the previous breastfeed as a full breast expression or first 2 ounces [34]; pooled samples from 2

220 breastfeeds if milk volume was low [12]. Collection of the samples throughout the day also varied, with 4
221 studies reporting morning collections [24, 36, 38, 41] and 16 not specifying the time. In 3 cohorts HM was
222 collected using electric breast pump [19, 24, 25, 41] while others used manual expression [10, 11, 21, 32, 35, 38] or
223 collected the drip from contra-lateral breast during a breastfeeding session [35, 41].
224
225 Upon collection the samples were either immediately frozen [21, 32] or maintained at room temperature from
226 30 min up to 1 [24, 25, 30], 4 [36, 38] and 12 hours [35, 40] until transported to the laboratory, with most of the studies
227 not fully specifying the storage/transit conditions. Identified differences in HM samples preparation
228 methodology included: centrifugation of the samples before [10, 19, 24, 25, 32-35, 38, 41] or after freezing [6, 11-13, 21,
229 29-31, 39, 40] and differences in acid treatment of the samples.
230
231 ***Measurement of TGF- β in human milk***
232 Sixteen of 21 studies used enzyme-linked immunosorbent assay (ELISA) to quantify TGF-β levels in HM.
233 Other techniques included: immunoassay [12], multiplex assay [13], custom-made multiplex assays [33], electro-
234 chemiluminescence [35] and quantikine immunoassay [40]. There was a considerable heterogeneity in reported
235 TGF-β concentrations, which made quantitative synthesis (meta-analysis) not possible. Lower levels of
236 detection for TGF-β1 differed among the studies varying from 7 pg/ml [41] to 30 pg/ml [30, 36, 38] and 60 pg/ml
237 [21, 39] using ELISA, while detection levels for TGF-β2 were 60 pg/ml using both ELISA [21, 39] and
238 immunoassay [12].
239
240 ***Statistical analyses***
241 Studies included into this systematic review can be classified based on statistical methods used to address
242 the research aim. Statistical approaches included: (a) univariate methods such as tests for groups
243 comparison and univariate analysis of the variance (ANOVA); (b) classical multivariate regression to
244 assess the association between risk factors and health outcome(s) adjusting for a number of confounders;

and (c) more advanced techniques such as least absolute shrinkage and selection operator (LASSO), principal components analysis (PCA) and causal mediation. Most of the studies performed univariate analysis with 15 studies carrying out only ANOVA and/or tests for group comparison such as t-test, chi-square test, Fisher's exact test and Mann-Whitney U-test. ANOVA was performed in 6 studies to assess time effect, treatment effect and/or the interaction effect on risk factors among groups. Ten studies performed multivariate logistic regression to estimate the association between risk factors and health outcome after adjusting for potential confounding factors, whilst 3 studies used more advance methods with 2 of them reporting detailed information on sensitivity analysis (**Table SII**). Eighteen studies did not report sample size power calculation, and none provided information on proportion of missing values among variables and methods used to handle missing values.

Some studies reported 'trends', not supported by the statistical analysis results (*P* - values > 0.05) [10, 21, 29] and only 3 studies [24, 25, 34] accounted for multiple comparisons. Half of the studies [10, 11, 13, 24, 25, 33-37] adjusted for potential confounders, including age, delivery mode, atopic status, length of breastfeeding, use of probiotics, site of collection, HM collection time, total storage time until analysis, introduction of food during the first year of life, paternal history of allergic diseases and presence of older siblings (**Table SII**).

Allergic health outcomes measured

Health outcomes were defined by clinical diagnosis [6, 10-13, 21, 29-32, 34, 38, 39], parental report [24, 35, 36, 41] and questionnaires with further clinical evaluation by the medical doctor [33, 37, 40] or based on well-validated instruments, such as "The International Study of Asthma and Allergies in Childhood" (ISAAC) study questionnaire [25], Hanifin and Rajka criteria [10, 29, 33] and UK Working party criteria [11-13, 30] for eczema. A single study obtained information on eczema diagnosis using both questionnaires with further evaluation by medical doctor, while wheezing was reported by parents [41].

Among health outcomes measured in this review, 16 (80%) population studies assessed the association between TGF-β concentration and risk of eczema, 9 (45%) asthma and/or wheezing, 6 (30%) food allergy development, 3 (15%) allergic rhinoconjunctivitis and 14 (70%) allergic sensitisation (**Tables I, SIII**).

Reference (year of publication)	N of participants/ milk samples •	Country	Population ^	TGF-β assessed and method used	Timing of sample collection	Age at outcome (months)	Health outcomes reported (method of assessment)	Overall effect*
Interventional studies								
Rautava 2002 [31]	62/NR	Finland	High risk	TGF-β1,2 (ELISA)	MM (3mo)	24	E, CMA (clinical history, DBPC, SPT), AS (SPT)	NS
Bottcher 2008 [10]	109/109	Sweden	High risk	TGF-β1,2 (ELISA)	C (3d) MM (1mo)	24	E (Hanifin and Rajka criteria), AS (SPT and/or sIgE)	– (AS)
Huurre 2008 [29]	140/NR	Finland	High risk	TGF-β2 (ELISA)	C (0d)	12	AS (SPT)	NS
Prescott 2008 [30]	105/105	New Zealand	High risk	TGF-β1 (ELISA)	C (3-7d) MM (1, 3mo)	24	E (UK Working party criteria), AS (SPT)	NS
Kuitunen 2012 [12]	NR/278 •	Finland	High risk	TGF-β2 (Immunoassay)	C (0-3d) MM (3mo)	60 ▪	FA (OFC); E (UK Working party criteria); A, AR (DD) ♦	– (E, AD)
Ismail 2013 [11]	79/79	Australia	High risk	TGF-β1 (ELISA)	TM (7d) MM (28d)	12	E (UK Working party criteria), AS (SPT), IgE-associated E (positive SPT)	NS
Simpson 2016[13]	259/259	Norway	Normal risk	TGF-β1-3 (Multiplex assay)	TM (10d) MM (3mo)	24	E (UK Working party criteria), AS (SPT and/or slgE)	NS
Observational studies								
Kalliomaki 1999[6]	47/43 •	Finland	Normal risk	TGF-β1,2 (ELISA)	C (0d) MM (3mo)	12	E (Hanifin and Rajka criteria)	+ (E)
Saarinen 1999 [39]	325/NR	Finland	Normal risk	TGF-β1 (ELISA)	C (1-4d)	3-13	CMA (Typical symptoms, SPT, sIgE), AS (positive SPT or sIgE)	+ (AS)
Bottcher 2003[32]	53/53	Sweden	Normal risk	TGF-β1,2 (ELISA)	C (0-4d) MM (1mo)	24	AS (SPT); A,E,ARC (DD) ♦	NS
Oddy 2003 [36]	243/142 •	USA	Normal risk	TGF-β1 (ELISA)	TM (14d)	12	W (Parental-reported)	+ (W)
Savilahti 2005 [40]	228/227 •	Finland	Normal risk	TGF-β1,2 (Quantikine Immunoassay)	C (1-4d)	48	CMA, E, A, AR, AC (questionnaire, paediatrician evaluation), AS (SPT, slgE)	NS
Rigotti 2006 [38]	22/22	Italy	Normal risk	TGF-β1 (ELISA)	C (3d) MM (1mo)	6	E (paediatrician evaluation)	NS
Snijders 2006 [41]	315/307 •	The Netherlands	Normal risk	TGF-β1 (ELISA)	MM (1mo)	24 ▪▪	E (ISAAC questionnaire, UK Working party criteria), W (Parental-reported), AS (sIgE)	NS
Tomicic 2010 [21]	99/99	Estonia, Sweden	Normal risk	TGF-β1,2 (ELISA)	C (0-4d) MM (1mo)	24	AS (SPT and/or slgE); E (DD)	NS

Soto-Ramirez 2012 [25] **2016** [24]	178/115	USA	Normal risk	TGF-β1 (ELISA)	MM (21d)	12	Scratching (questionnaires); asthma-like symptoms (ISAAC questionnaire)	+ (W)
Joseph 2014[34]	304/304	USA	Normal risk	TGF-β1 (ELISA)	MM (1mo)	36	FA (DD), AS (SPT, sIgE)	+ (AS)
Orivuori 2014 [37]	610/610	Finland, France, Germany, Switzerland	Normal risk	TGF-β1 (ELISA)	MM (2mo)	72 ▪▪▪	E and A (questionnaires, DD), AS (sIgE)	NS
Jepsen 2016 [33]	223/223	Denmark	High risk	TGF-β1 (custom-made Multiplex Assay)	MM (1mo)	36	E (Hanifin and Rajka criteria), W (daily diaries, DD)	NS
Munblit 2017[35]	398/315	UK, Italy, Russia	Normal risk	TGF-β1-3 (Electro-chemiluminescence)	C (0-6d) MM (1mo)	6	E, FA, W (Parental- reported), AS (SPT)	– (E)

Table I. Characteristics of the included studies A, asthma; AD, allergic diseases (cumulative outcome); AR, allergic rhinitis; ARC, allergic rhinoconjunctivitis; AS, allergic sensitization; C, colostrum (0-4 days) CMA, cow's milk allergy; D, days; DBPCFC, double-blind placebo-controlled food challenge; DD, doctor diagnosis; E, eczema; ELISA, enzyme-linked immunosorbent assay; FA, food allergy; ISAAC, International Study of Asthma and Allergies in Childhood; MM, mature milk (2 weeks and later); MO, months; NR, not reported; OFC, oral food challenge; SPT, skin prick test; sIgE, specific IgE levels; TGF-β, transforming growth factor-beta; TM, transitional milk (5-14 days); W, wheezing.
▪ SPT with common allergens and/or sIgE were assessed and associations between TGF-β and eczema reported up to 2 years; ▪▪ Eczema was evaluated up to 12 months; ▪▪▪ Eczema was evaluated up to 4 years. * overall effect of TGF-β1-3: "NS" - no significant effect; "+" - protective effect, "–" - higher risk of development of any reported allergic disease and/or allergic sensitization. ^ Children at high risk of allergy development were identified based on allergic history of mothers and/or family history. ♦ Number of analysed milk samples for TGF-β concentration unless not reported. ♦ Some allergic outcomes were combined for the purpose of statistical analyses.

TGF-β and development of allergic diseases

Overall, 60% of study populations in the review (12/20) showed no associations with HM TGF-β, 5/20 (25%) – protective effect and 3/20 (15%) – higher risk of allergy development (**Tables I and SIII; FIG.2**). TGF-β1 showed either no or some protective effect (5/18, 28%) on infant allergic outcomes, while conflicting results coming from TGF-β2 studies, with 3 studies (3/10, 30%) reporting high risk of allergy development and 1 (1/10, 10%) – protective effect of TGF-β2 **(FIG.3)**. TGF-β3 showed no associations with allergy development or allergic sensitisation [13, 35]. Five out of 15 individual health outcomes assessed at children below 2 years of age were associated with TGF-β in HM. Out of 30 individual health outcomes assessed at 2 years of age and beyond, only 3 were associated with the levels of TGF-β in HM (**FIG.2**).

FIG.2. Matrix of associations between TGF-β (cumulatively isoforms 1,2 and 3) in human milk and allergic outcomes at different age of outcome assessment. Horizontal lines indicate age of outcome assessment and vertical lines indicate allergic outcomes. Blue circle size indicates the total number of studies representing the matrix point and internal, smaller circles, indicate the number of studies showing positive (green circle)/negative (red circle) association between TGF-β and given allergic outcome, if available. Y.O. – years old, MO. – months.

Study	Eczema	Food allergy	Asthma/wheezing	Allergic rhinitis and/or conjunctivitis	Atopic sensitization
Interventional studies					
Rautava et al., 2002					
Bottcher et al., 2008					TGF-β2
Huurre et al., 2008					
Prescott et al., 2008					
Kuitunen et al., 2012 *	TGF-β2				
	TGF-β2				
Ismail et al., 2013					
Simpson et al., 2016					
Observational studies					
Kalliomaki et al., 1999	TGF-β1,2				
Saarinen et al., 1999					TGF-β1
Bottcher et al., 2003 *					
Oddy et al., 2003			TGF-β1		
Savilahti et al., 2005					
Rigotti et al., 2006					
Snijders et al., 2006					
Tomicic et al., 2010					
Soto-Ramirez et al., 2012, 2016			TGF-β1		
Joseph et al., 2014					TGF-β1
Orivuori et al., 2014					
Jepsen et al., 2016					
Munblit et al., 2017	TGF-β2				

High risk Protective effect No effect Not investigated Combined for analysis

FIG.3. Summary of associations between different isoforms of transforming growth factor beta in human milk and allergic outcomes in children. Colored boxes show significant positive (green) or negative (red) association between TGF-β and particular allergic outcome. * Allergic outcomes in this study were combined for the purpose of statistical analyses.

TGF-β and eczema

No consistent association was found between HM TGF-β and development of eczema. Among 15 studies [6, 10-13, 21, 24, 30, 31, 33, 35, 37, 38, 40, 41], only 2 reported higher risk of eczema development in children exposed to HM with higher TGF-β2 levels. According to Munblit *et al.*, infants receiving higher levels of mature

301 milk TGF-β2 were at higher risk (OR, 1.04; 95% CI, 1.01-1.06) of eczema development at 6 months [35].
302 Notably, Kuitunen *et al*. reported no association between TGF-β2 in colostrum and eczema, but higher
303 TGF-β2 concentration in mature milk was associated with eczema at 2 years of life [12]. For TGF-β1,
304 Kalliomaki *et al*. found higher concentrations in colostrum of mothers of infants with post-weaning onset
305 of eczema compared with those with no and pre-weaning onset of disease [6].
306
307 ***TGF-β and food allergy***
308 No association between TGF-β in HM and development of food allergy was reported [31, 34, 35, 39, 40], with
309 an exception of Saarinen *et al.* study, as authors reported concentration of TGF-β1 in colostrum of mothers
310 of infants with non IgE-mediated cow's milk allergy was significantly higher (mean 1162; 95% CI 881-
311 1531) pg/ml) than in IgE-mediated cow's milk allergy (589; 413-840) and healthy individuals (807; 677-
312 963 pg/ml) [39].
313 ***TGF-β and allergic sensitization***
314 Among 20 cohorts [10, 11, 13, 21, 29-32, 34, 35, 37, 39-41] 14 investigated relationship between HM TGF-β and allergic
315 sensitization with only two reporting protective effect and one associated with high risk. Bottcher *et al.*
316 found lower levels of TGF-β2 (<701 pg/ml) in colostrum of mothers of non-sensitized children at 24
317 months of age (OR, 0.3; 95% CI, 0.1-0.9), although higher levels (>1400 pg/ml) were not associated with
318 an increased risk of sensitization at 6 months of age (OR, 5.0; 95% CI, 0.9-27; P = .06) [10]. Saarinen *et al.*
319 [39] reported weak negative correlation between the concentration of colostrum TGF-β1 and SPT diameter
320 to cow's milk (r = -0.23; P = 0.02), β-lactoglobulin (r = -0.35, P = .01) and stimulation index to α-casein
321 (r = -0.28, P = .04) measured in infants with cow's milk allergy at the time of the challenge. In the study
322 of Joseph *et al*., among non-atopic mothers HM TGF-β1 concentrations were lower for those infants
323 classified as allergen-specific sIgE (1347, 1134-1600 vs. 1651, 1427-1910 pg/ml respectively, P = .047)
324 but among atopic mothers concentrations for these infants were higher (2161, 1868-2499 vs. 1525, 1347-

1726 pg/ml, $P = .001$), with no significant difference in concentration when stratified by positive SPT [34]. Orivuori *et al.* reported no consistent results at 4 and 6 years, however, at 6 years adjusted logistic regression models for IgE cut-off of 3.5 kU/l, but not for 0.35 and 0.7 kU/l, showed a significant difference (aOR, 95% CI : Q1, 0.40, 0.18-0.90, $P < 0.05$; Q2, 0.26, 0.11-0.61, $P < 0.01$) [37].

TGF-β and asthma/recurrent wheezing

Seven studies investigated associations between HM TGF-β and either asthma [37, 40] or recurrent wheezing [25, 33, 35, 36, 41]. Most of the studies reported no significant findings with only 2 detecting a protective effect of TGF-β1. In adjusted analyses, Soto-Ramirez *et al.* found that infants exposed to higher levels of TGF-β1 in mature HM had a lower risk of development of asthma-like symptoms at 6 and 12 months (RR = 0.31 (0.13-0.76) and 0.26 ($P = 0.01$) respectively) [25]. Similarly, Oddy *et al*. found a smaller percentage of wheeze in infants that received a higher dose of TGF-β1 through transitional HM ($P = 0.02$) [36].

TGF-β and allergic rhinitis/allergic rhinoconjunctivitis

The association between HM TGF-β levels and allergic rhinitis and/or allergic rhinoconjunctivitis was assessed in one cohort only, with no significant associations found [40].

TGF-β and immunological outcomes

Although our systematic review is focused on allergic outcomes, the initial search included search terms for immunological outcomes, so we provide an overview of the existing studies approaching this topic. Ogawa *et. al.* studied associations between TGF-β1 and TGF-β2 in colostrum of healthy mothers and serum IgA in newborns during the first month of life [15]. Notably, an increase of serum IgA in infants from birth to 1 month of life correlated with levels of both TGF-β1 ($r = 0.38$, $P = .005$) and TGF-β2 ($r = 0.45$, $P < 0.001$), while increase of IgM marginally correlated only with TGF-β2 ($r = 0.28$, $P = 0.04$) suggesting

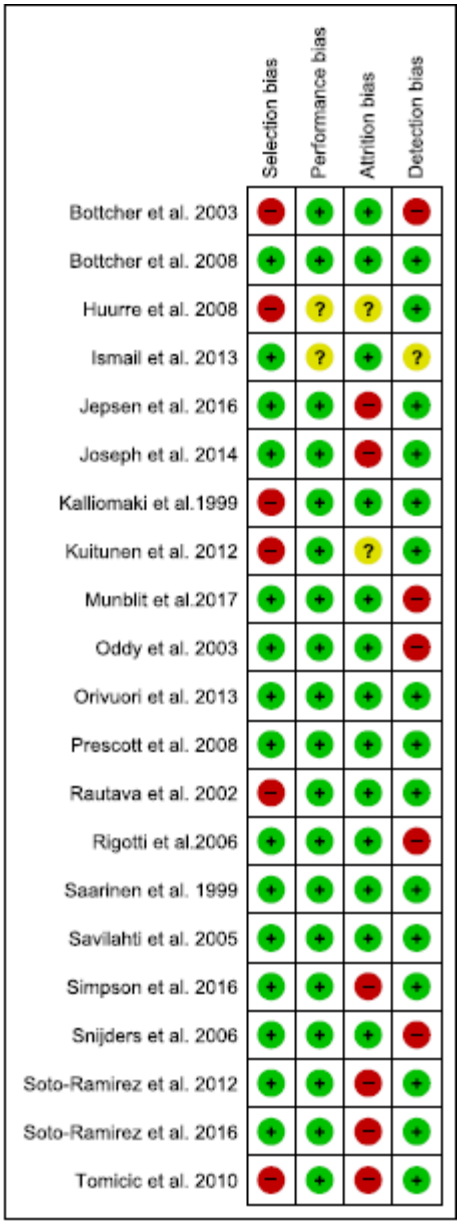
that colostral TGF-β may serve as the stimulus for IgA production in newborn infants. In line with this notion, Saarinen *et al.* found a positive correlation between TGF-β1 in colostrum and both, IgA antibodies to β–lactoglobulin (r = 0.204, *P* = 0.04) and IgG antibodies to α-casein (r = 0.237, *P* = 0.02) in infants prone to CMA [39]. Moreover, the size of SPT to CM (r = -0.228, *P* = 0.02) was negatively associated with the level of TGF-β1, indicating that TGF-β1 may inhibit IgE-mediated reactions to CM. Comparatively, TGF-β2 concentration in colostrum has been reported to associate with specific IgA responses to dietary antigens at 3 months of age (*P* = 0.048) [6]. In contrast, Prokesova *et. al*. investigated changes in the immune system of children genetically predisposed to allergic diseases reporting no significant differences in concentrations of TGF-β in colostrum and mature HM of allergic mothers compared with non-allergic mothers [14]. Although differences in concentrations of serum cytokines (IL-4, IL-10, IFN-γ) between the groups of healthy and high risk infants were reported, no analysis was conducted to test for associations between concentrations of HM TGF-β and these immunological outcomes [14]. The latter study, while reporting TGF-β concentrations in mature HM beyond the first month of lactation, did not differentiate between TGF-β isoforms. Difference in methodology, study design and immunological outcomes assessed does not allow for an appropriate analysis of these data.

Risk of bias

All the studies included in this systematic review were evaluated for their quality, with most being of a medium quality **(FIG.3).** High risk of bias was detected in 15 studies (71%). The main issues identified were attrition, selection and detection bias, including lack of adjustment for potential confounders and the use of self-reported questionnaires as a tool for allergic outcomes assessment.

It is worth noting that asthma diagnosis at the age below 3 years was considered an unreliable outcome as most children with “early wheeze” do not develop subsequent asthma [42]. We also considered any allergic outcome based on non-validated questionnaires without following clinical examination by doctor as a high risk of bias. We acknowledged any adjustments for known confounders performed by the authors,

but there is no clear guidance which adjustments are imperative. Attrition bias was calculated for all participants as it is not always clear if the same participants provided samples and completed the follow up; high attrition bias was considered as ≤75%.



376

377 **Fig.3** Risk of bias in studies assessing association between TGF-β concentration in human milk and allergic outcomes using
378 the National Institute for Clinical Excellence methodological checklist. '+, green', low risk of bias; '-, red', high risk of bias;
379 '?, yellow', unclear risk of bias.

Discussion

In this systematic review, we summarised findings of 21 studies from 20 cohorts of associations between HM TGF-β and allergic outcomes in infancy and childhood. Data from included studies showed no strong association between any isoform of TGF-β in HM and atopy/allergy development. Twelve new studies were identified [10-13, 21, 24, 25, 29, 33-35, 37] since the last systematic review[19], but evidence remains limited, due to high heterogeneity between studies, which makes any quantitative synthesis impossible. We used robust methods to search and synthesize evidence, provided critical analysis, identified several key strengths and limitations in the current literature and highlighted the unmet needs. In this paper, we provide a comprehensive overview of relationships between the levels of TGF-β in HM and allergic outcomes.

Although it was not possible to perform a meta-analysis, qualitative synthesis suggests that there is insufficient evidence of TGF-β influence on atopy/allergy development. Out of 15 studies only 3 found significant associations between TGF-β and eczema, 3 out of 14 with allergic sensitisation, 2 out of 9 with wheezing/asthma and none out of 5 linked TGF-β with IgE-mediated food allergy development. These results, however, do not completely exclude the possibility of TGF-β to impact allergic diseases development as a protective or negative effect was reported in some studies, with TGF-β1 being predominantly related to a protective effect and TGF-β2 associating with a higher risk of allergic disease. Studies differed in methods applied and outcomes reported and in addition to methodological heterogeneity, most of the studies carried a high risk of bias, thus results should be interpreted with caution.

Across a limited number of studies suggesting associations between the levels of HM TGF-β and allergic outcomes, opposite effects are generally reported for TGF-β1 and TGF-β2 isoforms. TGF-β1 is mainly associated with the protective effect, while TGF-β2 is linked with a higher risk of atopy/allergy development. There is increasingly more evidence, suggesting that TGF-β acts as a bi-functional

regulator, with its context-dependent nature of activities confirmed in a variety of biological responses and cell systems [43]. All TGF-β isoforms share a characteristic structure and are highly pleiotropic, but each isoform is linked with specific functions, therefore, may exert different effects. While TGF-β2 presents in much higher concentrations, accounting for up to 95% of TGF-β in HM, it is less potent than TGF-β1 [44-46]. TGF-β1-deficient mice were linked with the neonatal inflammatory disease [47], whereas TGF-β2,3-deficient mice present with developmental defects [48, 49]. This indicates that HM TGF-β isoforms may indeed have differential effects on allergy development.

HM contains a plethora of immune moderators [50], which may have synergistic and/or antagonistic effects to TGF-β, thus subsequently influencing the risk of atopy/allergy development. A recent study has investigated the effect of the immunological milieu of HM including 28 cytokines, chemokines and growth factors on development of cow's milk allergy. The authors reported interactions between the immune markers and showed that networks of HM regulatory and pro-inflammatory cytokines including TGF-β1, IL-1β, IL-6 and IL-10 are associated with tolerance to cow's milk development [51]. These findings suggest that narrowing research to single components could result in conflicting and even misleading findings and suggests that HM studies should implement a more holistic approach given the links between development of the immune system and both the gut microbiome [52] and HM oligosaccharides (HMO) [53].

We found a high degree of heterogeneity between studies, with differences in methodology (sample collection, storage and processing), populations (general population and high risk), outcome definition, age at outcome assessment, and approaches to statistical analysis being the most important contributors. Lack of standardized protocols of HM sample collection, storage and processing is an important issue, influencing the quality of HM research [54], associated with heterogeneity and not allowing for quantitative synthesis.

Previous research linked a number of physiological and environmental factors with the changes of TGF-β levels in HM. Stage of lactation and time of sample collection [9], circadian and seasonal variations [55, 56], time prior to freezing and length of sample storage [57], differences in laboratory techniques [55], ethnicity [34], residency [21], maternal lifestyle [35], smoking [37], diet [58], infection [59] as well as depression and anxiety [56, 60] were found to be a confounders and appear to impact TGF-β concentrations, thus possibly impacting health outcome development. Very few studies collected sufficient information and accounted for the duration and exclusivity of breastfeeding. Absence of this information prevents in-depth analysis of the dose-dependent effect. Although examination of associations between colostrum concentrations of HM components and infant outcomes is usually straightforward, the investigation of relationships with concentrations in mature HM is more problematic, given the variability in breastfeeding patterns and volumes of HM consumed during the lactation [61]. Our systematic review highlights pitfalls in HM research, with results not being data adjusted for known confounding factors in many studies up to date with only a few using multivariate statistical analysis (Table SII).

Differences in statistical methods applied largely contributed to the results of this review, with a large variety of strategies employed, yet lacking comprehensive approach and consistency. The issues associated with carrying out multiple hypothesis tests were rarely considered leading to a high risk of false-positive results and missing data was common but not dealt with, possibly resulting in under- and/or over-estimation of association between the exposure and outcome [62]. Adjustment for potentially important confounding factors may play an important part in identifying associations between levels of TGF-β and infant allergic outcomes but 6 studies did not report any adjustment. With heterogeneity in sample collection and processing, another important factor making meta-analysis impossible was data reporting, with some presenting adjusted and others non-adjusted data. In some studies data was not reported,

particularly if associations were not found to be significant. The different designs of the studies, e.g. observational and interventional, make it difficult to compare the results, as potential influence of the intervention cannot be excluded.

Health outcome definitions significantly varied between the studies contributing to heterogeneity. Some studies used less reliable measurements such as infant itchy rash [35], scratching [24] or asthma-like symptoms [25]. Parental-reported allergic outcomes are not always accurate and the possibility of overdiagnosis cannot be ruled out. It has been previously shown that mothers tend to over-report eczema in their children [63]. It should be noted, however, that most of the studies verified parental reports by physician assessment or used well-validated tools, such as the ISAAC questionnaire [64] or eczema UK Working party criteria [65]. There is also a marked difference in the age of health outcome assessment across the studies, ranging between 6 months and 6 years of age. Thirty percent of individual health outcomes assessed in children below 2 years of age were associated with TGF-β in HM, while only 3 out of 30 health outcomes at 2 years of age and beyond were found to be linked with TGF-β levels. This makes impact of HM TGF-β on allergic outcomes improbable and even if it exists in selected populations, it is highly unlikely that it extends beyond 2 years of age.

Atopic march theory suggests that allergic diseases progress from inflammatory skin manifestations, such as eczema during infancy, to asthma and allergic rhinitis in later childhood [66]. It has been demonstrated that many “early wheezers” do not subsequently develop persistent asthma [42]. There may even be an inverse relationship between early infection-induced wheeze and subsequent asthma [67]. It should be noted that most of the studies assessing asthma included in this systematic review, were realistically measuring wheeze rather than asthma, as age at health outcome assessment does not allow for appropriate asthma diagnosis. Apart from the Orivuori et al. diagnosing asthma at 6 years of life [37], no other study measured

this health outcome beyond the age of 4 years. Considering lack of studies reporting doctor's diagnosed health outcomes there is a need in further prospective cohorts, using well-validated instruments and standardized definitions, assessments of allergic outcomes and a considerable follow-up to evaluate the persistency of allergic symptoms.

The most recent systematic review [19] provided an overall measure of the effect of HM TGF-β on immunological outcomes in infants and children and reported that 8 out of 12 studies showed a positive association between either TGF-β1 or TGF-β2 concentrations and a reduction in allergy-related outcomes. These results are in conflict with our findings, which may be explained by the difference in systematic review inclusion criteria. Oddy and Rosales reviewed all the studies reporting any immunological, biochemical and/or clinical outcomes, including those assessing associations between maternal allergic status and HM TGF-β concentration, while this paper reviews associations between HM TGF-β and atopy/allergy development in offspring only.

Conclusion

TGF-β is an important immunological factor involved in inflammation regulation. Biological effects of HM TGF-β on allergic outcomes during infancy and childhood need to be further elucidated. Although several associations have been observed between HM TGF-β and allergic outcomes, our updated systematic review did not find strong evidence of association between the levels of TGF-β in HM and atopy/allergy development. Studies would benefit from an investigation into any dose-dependent effect, with an apparent lack of studies measuring exact amounts of breast milk consumed, in addition to immuno-active molecules measurement. Future studies should employ standardised, validated methods, accurate phenotyping of outcomes, use of comprehensive and consistent statistical methods to enable

meta-analyses. Implementation of a more holistic approach, assessing multiple immune markers level, HMO and microbiome would improve the quality of the research in the field.

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TABLE S1. Search strategies.

Embase Classic+Embase <1947 to 2017 June 7> (via Ovid)

1 allergy/ or hypersensitivity/ or immunoglobulin/ or immunoglobulin a/ or immunoglobulin a1/ or immunoglobulin a2/ or immunoglobulin e/ or secretory immunoglobulin/

2 (allergy or allergic diseases or allerg* or immun* outcomes or eczema or atopic dermatitis or itchy rash or allergic rhinitis or hay fever or food allergy or food hypersensitivity or asthma or wheeze or respiratory hypersensitivity or eosinophilic esophagitis or Ig serum level or immunoglobin blood level or immunoglobulin level or immunoglobulin serum level or plasma immunoglobulin or serum gammaglobulin or serum immune globulin or IgA or IgA1 or IgA2 or immunoglobulin A or IgE or immunoglobulin E or secretory immunoglobulin).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word]

3 food allergy/ or hypersensitivity/

4 asthma/ or allergic asthma/

5 wheezing/

6 eczema/ or dermatitis/

7 1 or 2 or 3 or 4 or 5 or 6

8 transforming growth factor/ or transforming growth factor beta/

9 (Transforming growth factor beta or transforming growth factor beta1 or transforming growth factor beta2 or transforming growth factor beta3 or TGF beta or TGFbeta or TGF-beta or TGF*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word]

10 8 or 9

11 colostrum/

12 human milk.mp. or breast milk/

13 (breast milk or breast milks or human milk or milk or breast milk human or mature milk or colostrum or early milk or transitional milk).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word]

14 11 or 12 or 13

15 (child* or infant* or boy* or girl* or newborn*).mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word] (3306386)

16 child/

17 infant/

18 newborn/

19 15 or 16 or 17 or 18

20 7 and 10 and 14 and 19

Cochrane library

1. "allergy" or allergic diseases or allerg* or immun* outcomes or "eczema" or "atopic dermatitis" or itchy rash or "allergic rhinitis" or "hay fever" or "food allergy" or food hypersensitivity or "asthma" or "wheeze" or respiratory hypersensitivity or eosinophilic esophagitis or Ig serum level or immunoglobin blood level or "immunoglobulin" or immunoglobulin level or immunoglobulin serum level or plasma immunoglobulin or serum gammaglobulin or serum immune globulin or "IgA" or IgA1 or IgA2 or "immunoglobulin A" or "IgE" or "immunoglobulin E"
2. "Transforming growth factor beta" or "transforming growth factor beta 1" or "transforming growth factor beta 1 level" or "transforming growth factor beta1" or "transforming growth factor beta 2" or "transforming growth factor beta 3" or TGF beta or TGFbeta or TGF-beta or TGF*
3. "breast milk" or breast milks or human milk or milk or breast milk human or colostrum* or "colostrum" or mature milk or transitional milk
4. child* or "child" or infant* or "infant" or boy* or girl* or newborn* or "newborn"
5. #1 and #2 and #3 and #4

Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid MEDLINE(R) <1946 to Present>

1 hypersensitivity/ or conjunctivitis, allergic/ or dermatitis, atopic/ or eosinophilic esophagitis/ or food hypersensitivity/ or respiratory hypersensitivity/ or asthma/ or rhinitis, allergic/ or urticaria/ or immunoglobulins/ or serum globulins/ or Immunoglobulin E/ or blood immunoglobulins.mp. or immunoglobulin a.mp. or Immunoglobulin A/

2 (allergy or allergic diseases or allerg* or immun* outcomes or eczema or atopic dermatitis or itchy rash or allergic rhinitis or hay fever or food allergy or food hypersensitivity or asthma or wheeze or respiratory hypersensitivity or eosinophilic esophagitis or Ig serum level or immunoglobin blood level or immunoglobulin level or immunoglobulin serum level or plasma immunoglobulin or serum gammaglobulin or serum immune globulin or IgA or IgA1 or IgA2 or immunoglobulin A or IgE or immunoglobulin E).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

3 1 or 2

4 transforming growth factor beta/ or transforming growth factor beta1/ or transforming growth factor beta2/ or transforming growth factor beta3/

5 (Transforming growth factor beta or transforming growth factor beta1 or transforming growth factor beta2 or transforming growth factor beta3 or TGF beta or TGFbeta or TGF-beta or TGF*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

6 4 or 5

7 Milk, Human/ or Colostrum.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

8 (breast milk or breast milks or human milk or milk or breast milk human or colostrum* or mature milk or transitional milk).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

9 7 or 8

10 adolescent/ or child/ or child, preschool/ or infant/ or infant, newborn/ or infant, low birth weight/ or infant, postmature/ or infant, premature/

11 (child* or infant* or boy* or girl* or newborn*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]

12 10 or 11

13 3 and 6 and 9 and 12

TABLE SII. Statistical analyses of association TGF- β in HM and allergic outcomes

Study	Statistical analyses of association between groups and between TGF-β in HM and allergic outcomes	Data expression and outcomes reported	Data transformation and adjustment to potential confounders
	Interventional studies		
Rautava 2002 [31]	The Student t-test for comparison of values between groups for normally distributed data. Mann-Whitney U-test for comparisons between groups for data of skewed distribution. The Chi-squared test for comparisons of proportions between groups.	Mean (95%CI) Median (IQR)	NR
Bottcher 2008 [10]	Mann-Whitney U-test for unpaired analyses. Spearman's rank order correlation coefficient test for correlations analyses. Multiple logistic regression and ANOVA for analyses of multivariate relationships.	Mean (range) OR (95% CI) *r*	Study treatment (placebo or *L. reuteri*) Maternal atopy Na/K ratios
Huurre 2008 [29]	Mann-Whitney U-test, w2 test, the t-test for the baseline and clinical characteristics. The association between infant sensitization and TGF-β tertiles (T1, T2 and T3) was given descriptively.	GM (95% CI) Probiotic/placebo ratios (95% CI)	* Logarithmically transformed NR
Prescott 2008 [30]	Mann-Whitney test for differences between the groups for non-parametric data. Chi-square or Fisher's exact test for differences between the groups for dichotomous data. Spearman or Kendall's Tau for correlations.	Median (IQR) τ	Factors with potential confounding effects were tested by correlation analyses. NR
Kuitunen 2012 [12]	Mantel-Haenszel method for the association between HM variables and allergic outcomes by the ages of 2 and 5 years in the treatment groups separately. The Breslow-Day test for evaluation whether the association is different in the probiotic group from that of the placebo group.	GM (95% CI) OR (95% CI)	* Logarithmically transformed Assessed separately by treatment group if Breslow-Day test indicated interaction
Ismail 2013 [11]	The Student's t-test for normally distributed continuous data. Mann-Whitney U-test was for skewed data. Chi-squared test or Fisher's exact test for categorical data. Logistic regression analyses.	Mean (SEM) GM (95% CI) Median (IQR)	* Skewed data log10-transformed Treatment group Maternal allergic status
Simpson 2016 [13]	Wilcoxon matched-pairs signed-rank test for comparison of the concentration at 10 days and 3 months. Linear regression for effect of probiotic supplementation on TGF-β concentrations. Causal mediation analysis (paramed) for HM cytokines which were found to be altered by probiotic supplementation.	Median (IQR) RR	Maternal atopy Maternal smoking during the first year of life Presence of older siblings
	Observational studies		
Kalliomaki 1999 [6]	The Fisher exact test or chi-squared test for differences in contingency tables. The Kruskal-Wallis test for comparisons between the groups. The Mann-Whitney U-test and Wilcoxon signed-rank test for comparisons between 2 unpaired and paired groups, respectively. Spearman rank correlation for correlation between 2 variables.	Median (IQR) % *r*	NR
Saarinen 1999 [39]	ANOVA was used for multiple comparisons. 2-tailed Student's t-test for comparisons between groups. Spearman rank correlation test for correlations between measurement from colostrum samples and from infants with CMA.	Mean (95%CI) *r*	* Logarithmically transformed Maternal atopy tested, NS

Bottcher 2003 [32]	Mann-Whitney U-test rank test for groups comparison. Spearman's rank order correlation coefficient test for correlations. The chi-square test for categorical variables, and Fisher's exact test for when the expected frequency for any cell was less than 5.	Median, Frequency (%) in groups	NR
Oddy 2003 [36]	Spearman nonparametric tests for correlation coefficients between cytokines. Contingency tables and the chi-square test for relationship between breastfeeding and wheeze in the first year of life. The relationship of the concentration of each cytokine in milk to wheeze was assessed by dividing the concentrations into tertiles with trend chi-square tests. Logistic regression analyses to determine the odds for any wheeze associated with cytokine dose in milk. A final categorization of concentration and duration of breastfeeding was calculated to reflect short or long duration of feeding (divided at the median in weeks) and low vs. medium-high cytokine concentration.	Mean (95%CI) OR (95%CI) *r*	Maternal characteristics: smoking at 1 year (any vs. none), education (≤12 vs. >12 years), history of physician-diagnosed asthma (yes vs. no) Offspring characteristics: sex, gestational age (<37 vs. ≥37 weeks), birth weight (≤6 lbs vs. >6 lbs), exposure to other children (presence of any older siblings or attendance at daycare with other children before 3 months)
Savilahti 2005 [40]	Multivariate stepwise logistic regression analysis by the forward selection method. Associations with symptoms of atopy and verified atopy were studied among the whole study group, those with long or short breast-feeding, and those with or without family history for atopy.	GM (95% CI) OR (95%CI)	* Logarithmically transformed NR
Rigotti 2006 [38]	Independent samples t-test for normally distributed variables and the Mann–Whitney U-test for variables not normally distributed for the differences between two unpaired groups.	Median (range)	The two groups of mothers were comparable for aging, alimentary habits, and pregnancy course. NR
Snijders 2006 [41]	ANOVA for comparisons of concentrations between groups. Logistic regression analysis for association between cytokines and infant's atopic manifestations. Extreme values of concentrations of cytokines were not excluded as these did not influence the results.	Mean (SD) OR (95%CI)	Recruitment group (conventional vs. alternative) Time interval between birth and HM collection (days) Total storage time in freezer until analysis (days) Maternal characteristics: age, allergic history, season of breast milk collection, use of probiotics, infection (during week of HM collection) Offspring characteristics: number of older siblings
Tomicic 2010 [21]	Mann-Whitney U test for unpaired analyses. Spearman's rank-order correlation coefficient test for correlation analyses. The 2-test for categorical variables, and the Fisher's exact test for when the expected frequency for any cell was 5.	Median (range) *rho*	NR
Soto-Ramirez 2012 [25]	Intra-class and Spearman correlation for associations of serum and whey immune markers. Log-linear regression for associations between immune markers and asthma-like symptoms at age 6 months and ever asthma-like symptoms in the first year of life (supplemental material). GEE was applied to predict repeated occurrence of asthma-like symptoms in infants at ages 6 and 12 months. In addition, models excluding infants who had both wheezy bronchitis and asthma-like symptoms were also ran.	RR (95%CI)	Maternal characteristics: race, age at pregnancy, smoking during pregnancy, household cigarette use at ages 6 and 12 months, maternal history of asthma, eczema, rhinitis, consumption of antibiotics during pregnancy, vaginal infections/pelvic conditions during pregnancy Offspring characteristics: gender, any respiratory infections at ages 6 and 12 months, season of birth ** FDR adjustment
Joseph 2014 [34]	Chi-square tests for subgroup comparisons of participant characteristics for binary and categorical variables. Wilcoxon Rank Sum (WRS) test cytokine levels comparison. Student's t-tests for comparison of continuous variables. Logistic regression model for each atopic phenotype with the log-transformed TGFβ1 values and the variable of interest (infant race/ethnicity or maternal atopy), along with the interaction term.	GM (95% CI) OR (95%CI)	* Logarithmically transformed Maternal characteristics: atopic status Offspring characteristics: race/ethnicity

Orivuori 2014 [37]	Linear regression for association between levels of HM TGF-β1 and exposures occurring up to month 2 of age. Uni-/multivariate smoothed plots based on generalized additive regression modelling for graphical display of significant associations of dose variables and health outcomes.	GM (95% CI) Quintiles	* Logarithmically transformed Centre as fixed effect, general farming variable (living on a farm vs. not), multiplicative interaction terms, breastfeeding duration as a confounder and as a multiplicative interaction term, TGF- β1 dose variable
Jepsen 2016 [33]	Cox regression for the association between cytokine levels with age at onset of eczema and recurrent wheeze during the first 3 years of life. Principal component analysis (PCA) was used to decompose the complex data set into fewer dimensions, reflecting the immunological intermediator correlation structure, and to extract patterns that describe the predominant variations in HM immune mediator levels. To avoid the effect of reverse causality, a sensitivity analysis was performed excluding all children with eczema diagnosis before end of exclusive breastfeeding.	HR (95% CI)	* Logarithmically transformed Delivery mode Household income Maternal eczema history Filaggrin mutation Household dog at birth and exclusive breastfeeding length ** FDR adjustment
Soto-Ramirez 2016 [24]	Ordinal logistic regression for the effect of probiotics. Wilcoxon matched-pairs signed-rank test for comparison of concentrations at 2 time points. Linear regression for effect of probiotic supplementation on TGF-β concentrations. Causal mediation analysis (paramed) for breast milk cytokines which were found to be altered by probiotic supplementation. GEE adjusting for within-participant effects using the regular maximum likelihood method for determination of the role of TGF-β in scratching at ages 6 and 12 months.	Median (IQR) OR (95%CI) RR (95%CI)	* Logarithmically transformed Maternal characteristics: race, age at pregnancy, marital status, smoking during pregnancy, household cigarette use at ages 6 and 12 months, maternal and paternal history of eczema, consumption of acetaminophen during pregnancy, and vaginal or urinary infections during pregnancy Offspring characteristics: gender and season of birth ** FDR adjustment
Munblit 2017 [35]	Univariate analysis and correlation matrix, followed by multivariate analysis which included modelling, using least absolute shrinkage and selection operator (LASSO) and generalized linear model (GLM).	Median (IQR) OR (95% CI)	* Binary variable-transformed (detectable vs. undetectable) Levels of growth factors in colostrum and HM, detectability of cytokines in colostrum and HM, site of collection, colostrum collection time, maternal atopic status, delivery type, infant gender

ANOVA, analysis of variance; CI, confidence interval; CMA, cow's milk allergy; FDR, false discovery rate; GEE, generalized estimating equation; GLM, generalized linear model; GM, geometric mean; HM, human milk; HR, hazard ratio; IQR, inter-quartile range; LASSO, least absolute shrinkage and selection operator; NR, not reported; OR, odds ratio; PCA, principal component analysis; RR, relative risk; SD, standard deviation; SEM, standard error of the mean; TGF-β, transforming growth factor beta; WRS, Wilcoxon Rank Sum.

TABLE SIII. Associations between TGF-β in human milk and allergic outcomes in children

Reference	Age at outcome assessment	TGF-β isoform and time of collection	Associations between human milk TGF-β and allergic outcomes
		Eczema (interventional studies)	
Rautava 2002 [31]	2 y.o	TGF-β1,2 (MM) 3 mo	NS (data not shown)
Bottcher 2008 [10]	2 y.o	TGF-β1,2 (C) 3d	NS (data not shown)
		TGF-β1,2 (MM) 1 mo	NS (data not shown)
Prescott 2008 [30]	2 y.o	TGF-β1 (C) 3-7d	NS (data not shown)
		TGF-β1 (MM) 1 and 3 mo	NS (data not shown)
Kuitunen 2012 [12]	2 y.o	TGF-β2 (C) 0-3d	NS: OR, 1.05; 95% CI ,0.61-1.79
	Atopic E 2 y.o		NS: OR, 1.16; 95% CI, 0.57-2.34
	2 y.o	TGF-β2 (MM) 3 mo	**↑TGF-β2 (MM) - ↑higher risk of E by the age of 2 years (OR, 2.30; 95% CI, 1.34-3.94)**
	Atopic E 2 y.o		NS: OR, 1.51; 95% CI, 0.77-2.96
Ismail 2013 [11]	1 y.o	TGF-β1 (TM) 7 d	No E (451.3 (330.4725.4)) vs. E (450.4 (357.2798.7)): NS, *P* = 0.7; a*P* = .9
		TGF-β1 (MM) 28 d	No E (284.9 (200433.6)) vs. E (269.3 (200366.0): NS, *P* = 0.7; a*P* = 0.6
Simpson 2016 [13]	2 y.o	TGF β1-3 (TM) 10 d	NS (data not shown)
		TGF β1-3 (MM) 3 mo	NS (data not shown)
		Eczema (observational studies)	
Kalliomaki 1999 [6]	1 y.o	TGF-β1,2 (C) 0d	**↑TGF-β1 - ↑higher post-weaning onset of E compared with no onset (*P* = .0056) and pre-weaning onset (*P* = .0008);** **↑TGF-β2 - ↑higher post-weaning onset of E compared with pre-weaning onset (*P* = .015) and comparable to nonatopic control subjects.**
		TGF-β1,2 (MM) 3 mo	NS
Savilahti 2005 [40]	4 y.o	TGF-β1,2 (C) 1-4 d	NS (data not shown)
Snijders 2006 [41]	1 y.o	TGF-β1 (MM) 1 mo	NS: Low (2.0-166.9 pg/ml): aOR, 1.0; 95% CI, reference; *n* = 30 Middle (166.9-248.4 pg/ml): aOR, 1.14; 95% CI, 0.59-2.10; *n* = 32 High (248.5-1536.8): aOR, 1.00; 95% CI, 0.53-1.91; *n* = 28 *P*-value for trend: aOR, 1.00; 95% CI, *n* = 299
Rigotti 2006 [38]	6 mo	TGF-β1 (C) 3d TGF-β1 (MM) 1 mo	4/6 infants who developed E received milk with no TGF-β1 in both C and MM. No statistical analysis has been performed.
Tomicic 2010 [21]	2 y.o	TGF-β1,2 (C) 0-4 d TGF-β1,2 (MM) 1 mo	NS (data not shown)
Orivuori 2014 [37]	2 y.o	TGF-β1 (MM) 2 mo	NS: aOR, 0.86; 95% CI, 0.65-1.14.
			all NS (aOR, 95% CI): Q1, 1.00; Q2, 0.95, 0.48-1.90; Q3, 1.12, 0.58-2.16; Q4, 0.64, 0.30-1.34; Q5, 1.00, 0.50-1.98.
	4 y.o		NS: aOR, 0.83; 95% CI, 0.63-1.08.
			all NS (aOR, 95% CI): Q1, 1.00; Q2, 0.77, 0.40-1.51; Q3, 1.13, 0.60-2.12; Q4, 0.87, 0.45-1.71; Q5, 1.10, 0.57-2.09.
Jepsen 2016 [33]	3 y.o	TGF-β1 (MM) 1 mo	NS: HR 0.89; 95% CI, 0.72-1.10; *P* = 0.29; aHR 0.91; 95% CI, 0.74-1.12
Soto-Ramirez 2016 [24]	Scratching 1 y.o	TGF-β1 (MM) 21d	NS: TGF-β1 level ≥774.63: RR, 0.71; 95 % CI, 0.48-1.05

			Level 438.28–774.63: RR, 0.98; 95 % CI, 0.73-1.31 Level <436.28 - reference.
Munblit 2017 [35]	6 mo.	TGF-β2 (C) 0-6 d TGF-β2 (MM) 1 mo TGF-β3 (C) 0-6 d TGF-β3 (MM) 1 mo	NS: a*P* = 0.66 **↑TGFβ2 (MM) - ↑higher risk of E (aOR, 1.04; 95% CI, 1.01–1.06)** NS: a*P* = 0.087 NS: *aP* = 0.17 NS: a*P* = 0.66
Atopic sensitization (interventional studies)			
Rautava 2002 [31]	2 y.o	TGF-β1,2 (MM) 3 mo	NS (data not shown)
Bottcher 2008 [10]	2 y.o	TGF-β1 (C) 3 d ↓TGF-β2 (C) [<701**] TGF-β2 (C) [>1400] TGF-β1,2 (MM) 1 mo	NS (data not shown) **↓AS during first 2 years: OR, 0.3; 95% CI, 0.1-0.9** AS at 6 months: NS: aOR, 5.0; 95% CI, 0.9-27 NS (data not shown)
Huurre 2008 [29]	1 y.o	TGF-β2 (C) 0 d	NS (data not shown)
Prescott 2008 [30]	2 y.o	TGF-β1 (C) 3-7 d TGF-β1 (MM) 1 and 3 mo	NS (data not shown) NS (data not shown)
Ismail 2013 [11]	1 y.o	TGF-β1 (TM) 7 d TGF-β1 (MM) 28 d	NS (data not shown) NS (data not shown)
Simpson 2016 [13]	2 y.o	TGF β1-3 (TM) 10 d TGF β1-3 (MM) 3 mo	NS (data not shown) NS (data not shown)
Atopic sensitization (observational studies)			
Saarinen 1999 [39]	1 y.o	TGF-β1 (C) 1-4 d	**↑TGF-β1 (C) - ↓SPT to CM (r = -0.228, *P* = .02); ↓SI to α-casein (*r* = -0.282, *P* = .04) and ↓SI to β-lactoglobulin (*r* = -0.347, *P* = .01);** NS: sIgE to CM (*r* = -0.138, *P* = .18); SI to β-casein (*r* = -0.241, *P* = .08).
Bottcher 2003 [32]	2 y.o	TGF-β1,2 (C) 0-4 d TGF-β1,2 (MM)1 mo	NS (data not shown) NS (data not shown)
Savilahti 2005 [40]	4 y.o	TGF-β1,2 (C) 1-4 d	NS (data not shown)
Snijders 2006 [41]	2 y.o	TGF-β1 (MM) 1 mo	NS: Low (2.0-166.9 pg/ml): aOR, 1.0; 95% CI, reference; *n* = 17. Middle (166.9-248.4 pg/ml): aOR, 1.19; 95% CI, 0.52-2.74; *n* = 21. High (248.5-1536.8 pg/ml): aOR, 0.51; 95% CI, 0.21-1.24; *n* = 12. *P*-value for trend: aOR, 0.13; 95% CI, *n* = 200.
Tomicic 2010 [21]	2 y.o	TGF-β1,2 (C) 0-4 d	NS: In Sweden (median, 486; range, 240-1400 pg/mL, in non-sensitized vs. median 586; range, 365-1156, in sensitized infants; *P* = .11).
Joseph 2014 [34]	3 y.o	TGF-β1 (MM) 1 mo	**↓GM in non-atopic mothers of infants with elevated vs. not elevated sIgE (1347 vs. 1651 pg/ml respectively, *P* = .047)** **↑GM in atopic mothers of infants classified as allergen-specific IgE (2161 vs. 1525 pg/ml respectively, *P* = .001).**
Orivuori 2014 [37]	4 y.o 6 y.o	TGF-β1 (MM) 2 mo	NS: aOR, 1.10; 95% CI, 0.85-1.42. Based on IgE cut-off 0.35Ku/l: All NS (aOR, 95% CI): Q1, 1.00; Q2, 1.10, 0.60-2.01; Q3, 1.03, 0.56-1.91; Q4, 1.46, 0.79-2.70; Q5, 1.76, 0.93-3.32. All other cut-offs/ages: NS, *P* > .05. NS: aOR,1.05; 95%-CI, 0.82-1.36. Based on IgE cut-off 0.35Ku/l: All NS (aOR, 95% CI): Q1, 1.00; Q2, 0.75, 0.41-1.35; Q3, 1.04, 0.56-1.93; Q4, 0.84, 0.46-1.53; Q5, 1.02, 0.55-1.89. **Based on IgE cut-off 3.5 kU/l (aOR, 95% CI): Q1, 0.40, 0.18-0.90; Q2, 0.26, 0.11-0.61.** All other cut-offs/ages: NS, *P* > .05.
Munblit 2017 [35]	6 mo	TGF-β1-3 (C) 0-6 d TGF-β1-3 (MM) 1 mo	NS (data not shown)

Asthma/wheezing (observational studies)			
Oddy 2003 [36]	Asthma-like symptoms 1 y.o	TGF-β1 (TM) 14 d	NS (P = .18). **↑Dose of TGF-β1 (TM) - ↓lower risk of wheeze (%) (P = .017), relationship is linear (P = .006 trend χ2).** **↑ Dose of TGF-β1 (TM) - ↓lower risk of wheeze (OR, 0.22; 95% CI, 0.05-0.89).**
Savilahti 2005 [40]	A 4 y.o	TGF-β1,2 (C) 1-4 d	NS (data not shown)
Snijders 2006 [41]	W 2 y.o	TGF-β1 (MM) 1 mo	NS: Low (2.0-166.9 pg/ml): aOR, 1.0; 95% CI, reference; n = 24. Middle (166.9-248.4 pg/ml): aOR, 1.21; 95% CI, 0.61-2.93; n = 28. High (248.5-1536.8 pg/ml): aOR, 1.13; 95% CI, 0.57-2.23; n = 28. P-value for trend: aOR, 0.73; 95% CI, n = 299.
Soto-Ramirez 2012 [25]	Asthma-like symptoms 1 y.o	TGF-β1 (MM) 21 d	**↑TGF-β1 (MM, 4th and 3d quartile levels) - ↓lower risk of asthma-like symptoms (RR, 0.31; P = .01 and 0.26, P = .002 respectively).**
Orivuori 2014 [37]	A 4 y.o A 6 y.o	TGF-β1 (MM) 2 mo	NS: aOR, 0.77; 95% CI, 0.47-1.27. All NS (aOR, 95% CI): Q1, 1.00; Q2, 0.57, 0.18-1.84; Q3, 0.43, 0.12-1.50; Q4, 0.53, 0.15-1.87; Q5, 0.65, 0.21-2.02. NS: aOR, 0.92; 95% CI, 0.62-1.38. All NS(aOR, 95% CI):Q1, 1.00; Q2, 0.90, 0.37-2.20; Q3, 0.43, 0.15-1.24; Q4, 0.83, 0.32-2.13; Q5, 0.70, 0.27-1.82.
Jepsen 2016 [33]	W 3 y.o	TGF-β1 (MM) 1 mo	NS: HR, 0.91; 95% CI, 0.68-1.23; P = .54; aHR, 0.90; 95% CI, 0.66-1.22
Munblit 2017 [35]	W 6 mo	TGF-β2 (C) 0-6 d TGF-β2 (MM) 1 mo TGF-β3 (C) 0-6 d TGF-β3 (MM) 1 mo	NS: aP = 0.283 NS: aP = 0.090 NS: aP = 0.193 NS: aP = 0.283
Food allergy (interventional studies)			
Rautava 2002 [31]	CMA 2 y.o	TGF-β1,2 (MM) 3 mo	NS (data not shown)
Food allergy (observational studies)			
Saarinen 1999 [39]	CMA 1 y.o	TGF-β1 (C) 1-4 d	**↓TGF-β1 ($C_{\text{infants with later development of IgE-mediated CMA}}$) - ↑ non-IgE-mediated CMA (t = 2.57, P = .012).** The level of control subjects did not differ from both groups.
Savilahti 2005 [40]	CMA 4 y.o	TGF-β1,2 (C) 1-4 d	NS (data not shown)
Joseph 2014 [34]	3 y.o	TGF-β1 ($MM_{\text{Among non-black infants}}$) 1 mo	TGF-β1 ($MM_{\text{Among non-black infants}}$) - FA vs. no FA: NS ($P$ = .081); SPT+ $_{\text{food allergens}}$ vs. SPT-: NS (P = .064)
Munblit 2017 [35]	6 mo.	TGF-β2 (C) 0-6 d TGF-β2 (MM) 1 mo TGF-β3 (C) 0-6 d TGF-β3 (MM) 1 mo	NS: aP = 0.91 NS: aP = 0.98 NS: aP = 0.49 NS: aP = 0.91
Allergic rhinitis/conjunctivitis (observational studies)			
Savilahti 2005 [40]	ARC 4 y.o	TGF-β1,2 (C) 1-4 d	NS (data not shown)
Studies with combined allergic outcomes (interventional studies)			
Kuitunen 2012 ***[12]	AlD 2 y.o AlD 5 y.o AtD 2 y.o AtD 5 y.o AlD 2 y.o AtD 2 y.o AlD 5 y.o AtD 5 y.o	TGF-β2 (C) 0-3 d TGF-β2 (MM) 3 mo	NS: OR, 1.05; 95% CI, 0.62-1.76 NS: OR, 1.08; 95% CI, 0.66-1.77 NS: OR, 1.31; 95% CI, 0.66-2.62 NS: OR, 1.06; 95% CI, 0.63-1.79 **↑TGF-β2 (MM) - ↑higher risk of allergic disease by the age of 2 years (OR, 2.23; 95% CI, 1.33-3.74)** NS :OR, 1.74; 95% CI, 0.90-3.37 NS: OR, 1.36; 95% CI ,0.84-2.21 NS: OR, 1.04; 95% CI, 0.62-1.73
Studies with combined allergic outcomes (observational studies)			
Bottcher 2003 [32]	E 2 y.o	TGF-β1,2 (C) 0-4 d	

A 2 y.o ARC 2 y.o	TGF-β1,2 (MM) 1 mo	NS: the number of positive samples or the levels of the cytokines in C or MM and the development of either allergic symptom (*P*-values: .14 - .99).

A, asthma; AlD, allergic diseases; AtD, 'atopic' diseases; aHR, adjusted hazard ratio; aOR, adjusted odds ratio; a*P*, adjusted *P*-value; AR, allergic rhinitis; ARC, allergic rhinoconjunctivitis; AS, allergic sensitization; C, colostrum (0-4 days); CI, confidence interval; CM, cow's milk; CMA, cow's milk allergy; D, days; DNS, data not shown (not presented in the paper); E, eczema; FA, food allergy; FDR, false discovery rate (adjusted); GM, geometric mean; HR, hazard ratio; IQR, inter-quartile range; MA, multivariate analysis; MM, mature human milk (2 weeks and later); MO, months; NA, non-applicable; NR, not reported; NS, non-significant; OR, odds ratio; Q, quintile; RR, relative risk; SD, standard deviation; SI, stimulation index – expression of proliferation (median counts per minute incorporated in the presence of the antigen divided by median counts per minute incorporated in the absence of the antigen); SPT, skin prick test; sIgE, specific IgE levels; TGF-β, transforming growth factor beta; TM, transitional milk (5-14 days); W, wheezing.
"↑" – stands for increased and "↓" – stands for decreased levels or reduced risk of TGF-β or disease/parameter; * Age, up to which outcomes were assessed; ** All concentrations are pg/ml; *** This study analyzed eczema separately and also combined allergic diseases for the analysis (eczema, food allergy, allergic rhinitis and asthma) and atopic (IgE-associated) allergic diseases.